INHIBITION OF YEAST HYDROXYMETHYLGLUTARYL-CoA REDUCTASE BY A RAT LIVER MITOCHONDRIAL PREPARATION

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1. Introduction

The control of sterol formation has obvious significance both in nutrition and therapy. Hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), a rate regulating enzyme in the biosynthesis of sterols, is being examined intensively as a site for the control of sterol synthesis. A mitochondrial preparation (ICS) from the livers of starved rats inhibited the biosynthesis of cholesterol in biological tests both in vivo and in vitro [1–3]. This preparation also inhibited sterol synthesis in the mycelia of the fungus *Fusarium oxysporum* [4]. In addition Migicovsky [5] has demonstrated that ICS inhibited at a point in the sterol biosynthetic pathway prior to mevalonate formation in rat liver tissues. Since the sterol biosynthetic pathway to the stage of squalene is essentially the same in all organisms examined, yeast, which produced HMGCoA** reductase more abundantly than rat liver tissues, was used as the source of the enzyme. This report identifies HMGCoA reductase as the site of the action of the mitochondrial inhibitor of sterol synthesis.

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** *Reagent abbreviations:* Tris-(hydroxymethyl)-aminomethane (Tris); 3-hydroxy-3-methylglutaryl Coenzyme A (HMGCoA); mevalonic acid (MVA); glucose-6-phosphate (G6P); glucose-6-phosphate dehydrogenase (G6PD); oxidized triphosphopyridine nucleotide (TPN); triethanolamine (TEA) from Sigma Chemical Company; dithiothreitol (DTT) from NBC; 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) from Calbiochem, trichloroacetic acid (TCA) and ethelenediaminetetra acetate (EDTA) from Fisher Scientific Co.

2. Materials and methods

2.1. *Organism*

Brewer's yeast, *Saccharomyces cerevisiae,* was obtained from O'Keefe's Breweries, Montreal. The yeast, used in the preparation of ale from barley, was grown 15 hr aerobically and 45 hr anaerobically after carbon dioxide saturation of the fermenting vat.

2.2. *Preparation of yeast HMGCoA reductase*

Immediately upon receipt, the yeast cells were washed with glass distilled water in a large Buchner funnel. The cells were air-dried, quick-frozen in liquid nitrogen and stored in plastic bags at −20°C.

Cells (100 g) in 150 ml of cold 0.1 M triethanolamine buffer, pH 7.4, containing 0.02 M EDTA and 2 mM DTT were disrupted with 200 g of small glass beads in an Omnimix, for 8 min at high speed (100–110 setting on Powerstat, Superior Electric Co., Bristol, Conn.). Disruption of cells was done in 2 min periods in the cold.

The supernatant was poured off and the residue washed with a minimum amount of buffer three times. The washings were pooled with the supernatant and centrifuged twice at 12 000 *g* for 10 min (Sorvall RC2B). The supernatant solution was then centrifuged at 60 000 *g* for 60 min (Spinco Model L.).
The supernatant was discarded and the pellets in the centrifuge tubes were stored in the freezer. Fresh or frozen microsomes were resuspended in 0.1 M triethanolamine buffer at pH 7.4 containing 20 mM EDTA and 10 mM dithiothreitol and allowed to stand 1 hr at 4°C before centrifugation at 60 000 *g* for 45 min.

The pellet of microsomal protein was suspended

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in 2 ml of cold (4°C) buffer (0.1 M TEA, 0.2 M EDTA, 2 mM DTT) at pH 7.4. The samples of microsomal protein were diluted with buffer to give the final protein concentration for CoASH assay.

2.3. *Assay for MGCoA reductase activity*

The activity of the enzyme was determined by the amount of CoASH released from the substrate HMGCoA and was measured by the method of Hulcher and Oleson [8]. The monothiol was reacted with DTNB and the absorption was measured at 412 nm.

In these experiments one unit of enzyme (mU) is that amount catalyzing the turnover of 1 nmole of HMGCoA per minute at 37°C. Since 1 mole of HMGCoA produces one mole of CoHSH in the catalytic reaction [7], the units of enzyme were based on the CoASH equivalence.

2.4. *Preparation of the inhibitor*

The inhibitor was obtained from the mitochondria of starved rats' livers as described previously by Migicovsky [2]. The sucrose (0.25 M)-perfused livers were homogenized according to Bucher [8]. The homogenate was centrifuged at 700 *g* and the supernatant at 9000 *g* for 15 min. The mitochondrial pellet, dispersed in distilled water, was sonicated at 20 kcycles for 5 min at 4°C, then centrifuged at 105^3 *g* for 30 min. The supernatant was dialysed against distilled water for 96 hr in the cold then freeze dried. This preparation (I) was then digested for 1 hr with each of insolubilized trypsin† and β-chymotrypsin† (each 3.4 mg/g I) at pH 8.5. After removal of the digesting enzymes by filtration, the pH of the digest was reduced to 3 by adding 0.01 N HCl dropwise. The precipitate thus formed was washed and extracted with ethanol, which was finally removed by evaporation under nitrogen, leaving the residue of the alcohol extract II. Controls, omitting I, were prepared similarly. Other controls were distilled water and sucrose. The controls showed no inhibition. Protein was determined by the method of Lowry et al. [9].

† From bovine pancreas, Sigma Chemical Co., insolubilized by coupling to Sepharose 4B (Pharmacia Ltd.) by the cyanogen bromide method.

3. Results and discussion

Two mitochondrial preparations were examined for their inhibition of HMGCoA reductase. Preparation I, mitochondrial fragments containing 38% protein, inhibited the enzyme 51%. Preparation II, the ethanol extract of the acid precipitate of the digested fragments, contained 68% protein and inhibited the enzyme 89% (table 1). Digestion apparently released the inhibitor from the mitochondrial membranes. The supernatant, freeze-dried and tested after the pH 3 precipitation, showed no inhibition. Cayen and Dvornik have demonstrated post- and pre-mevalonate inhibitors of cholesterol synthesis obtained from similar mitochondrial preparations from rats [10].

Fig. 1 illustrates the dose-dependent inhibition of HMGCoA reductase obtained with the ethanol extract of the acid precipitate of the mitochondrial digest (II).

The MHGCoA reductase activity in these experiments was obtained from the microsomal fraction of the yeast. In baker's yeast, grown aerobically, the enzyme was solubilized by autolysis of the mitochondrial pellet [11] and was shown to be in the mitochondria obtained by differential centrifugation [12]. In bacteria the enzyme appears to be

Table 1
Inhibition of HMGCoA reductase by mitochondrial preparations

Inhibitor treatment (1 mg)	CoASH released nmoles/min*	% Inhibition***
No inhibitor	0.37**	0
Preparation I (sonicated mitochondrial fragments)	0.18	51
Preparation II (ethanol extract of pH 3 precipitate preparation I digested	0.04	89

* Reaction mixture: 100 μl yeast microsomal protein, 2 units G6PD, 2 μmoles TPN, 3 μmoles G6P, 150 nmoles HMGCoA, 0.2 mM arsenite, 0.2 μmoles DTT in a final volume of 1 ml.

** Enzyme preparation 0.37 mU, 0.28 mg protein/100 μl releasing 1.31 nmoles CoASH/min/mg protein.

*** Inhibition relative to inhibitor-free reaction. Four replicates per treatment. Differences between treatments significant at $p < 0.01$.

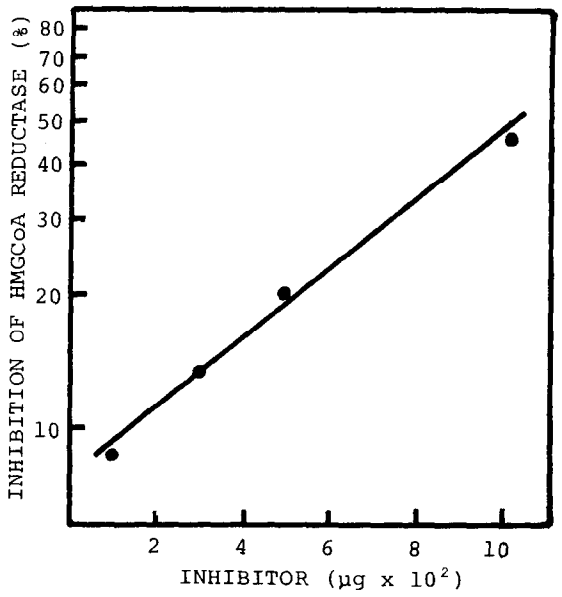

Fig. 1. Reaction mixture: Enzyme preparation (0.74 mU) 0.44 mg microsomal protein, 2 units G6PD, 2 µmoles TPN, 3 µmoles G6P 150 nmoles HMGCoA, 0.2 mM arsenite, 0.2 µmoles DTT in a final volume of 1 ml. Inhibitor: Ethanol extract of pH 3 precipitate.

soluble [13] and microsome-bound in rat and pigeon liver tissues [6,14,15]. In the present study, the anaerobic growth of the yeast cells, however, possibly altered mitochondrial formation such that HMGCoA reductase localization was reduced for eliminated from this site. There is also the possibility of the microsomal synthesis of HMGCoA reductase and its eventual transfer to the mitochondria analogous to that of glutamate dehydrogenase in rats' liver tissue described recently by Godinot and Lardy [16].

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